



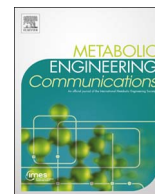
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Metabolic engineering of *Saccharomyces cerevisiae* for overproduction of triacylglycerols

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A B S T R A C T

Triacylglycerols (TAGs) are valuable versatile compounds that can be used as metabolites for nutrition and health, as well as feedstocks for biofuel production. Although *Saccharomyces cerevisiae* is the favored microbial cell factory for industrial production of biochemicals, it does not produce large amounts of lipids and TAGs comprise only ~1% of its cell dry weight. Here, we engineered *S. cerevisiae* to reorient its metabolism for overproduction of TAGs, by regulating lipid droplet associated-proteins involved in TAG synthesis and hydrolysis. We implemented a push-and-pull strategy by overexpressing genes encoding a deregulated acetyl-CoA carboxylase, ACC1^{S659A/S1157A} (ACC1**), as well as the last two steps of TAG formation: phosphatidic phosphatase (PAH1) and diacylglycerol acyltransferase (DGA1), ultimately leading to 129 mg-gCDW⁻¹ of TAGs. Disruption of TAG lipase genes TGL3, TGL4, TGL5 and sterol acyltransferase gene ARE1 increased the TAG content to 218 mg-gCDW⁻¹. Further disruption of the beta-oxidation by deletion of POX1, as well as glycerol-3-phosphate utilization through deletion of GUT2, did not affect TAGs levels. Finally, disruption of the peroxisomal fatty acyl-CoA transporter PXA1 led to accumulation of 254 mg-gCDW⁻¹. The TAG levels achieved here are the highest titer reported in *S. cerevisiae*, reaching 27.4% of the maximum theoretical yield in minimal medium with 2% glucose. This work shows the potential of using an industrially established and robust yeast species for high level lipid production.

1. Introduction

In recent years, there has been an increasing demand for oils with specialized properties. Most oils found in nature are mainly composed of triacylglycerols (TAGs). Natural producers with specific TAG compositions, such as exotic plants, are usually poorly understood at the metabolic level, and lack available tools for introducing genetic modification, which ultimately makes them difficult to engineer. Consequently, there is an important need for alternative organisms that can accumulate high lipid amounts and be easily engineered for tailored oil production.

Although certain microorganisms are known to accumulate high levels of lipids, *Saccharomyces cerevisiae* remains the preferred cell factory for production of biochemicals (Nielsen and Keasling, 2016; Krivoruchko and Nielsen, 2015). It is a Generally Regarded As Safe (GRAS) organism with high tolerance to low pH and fermentation inhibitors. One of the major advantages of *S. cerevisiae* is the vast

knowledge available about its physiology and metabolism, providing a solid background for engineering approaches (Julleson et al., 2015). It is the most tractable organism, with several available tools for genome engineering, such as the CRISPR-Cas system, which has proven to be highly efficient (Dicarlo et al., 2013; Jakočiūnas et al., 2015; Zalatan et al., 2015).

TAGs are composed of three fatty acid chains esterified with a glycerol backbone. As such, the first key step towards increasing the TAG production potential in *S. cerevisiae* is the upregulation of the fatty acid biosynthesis. Fatty acids are synthesized as acyl-CoAs by the fatty acid synthetase complex, encoded by the genes *FAS1* and *FAS2* (Schweizer et al., 1984). Malonyl-CoA is used in this process as a substrate for iterative elongation of acyl chains, and is synthesized from acetyl-CoA by the enzyme acetyl-CoA carboxylase (Acc1). This enzyme is tightly regulated at the transcriptional and protein-level, rendering it a major regulation step in fatty acid biosynthesis (Hasslacher et al., 1993; Blank et al., 2017; Bozaquel-Morais et al., 2017). It is inhibited by

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Snf1 phosphorylation, and the expression of a double mutant *Acc1*^{S659A/S1157A} with two removed phosphorylation sites, here shortened *Acc1*^{**}, has been shown to increase production of malonyl-CoA and fatty acid-derived products up to 3-fold compared to the wild-type (wt) strain (Shi et al., 2014).

The second point of focus is increasing the flux of acyl chains towards TAG formation. TAG assembly is initiated with glycerol-3-phosphate (Gro-3-P), which is consecutively acylated with two acyl-chains, by GPAT and LPAT genes, to form phosphatidic acid (PA). PA is then dephosphorylated by the phosphatidic acid phosphohydrolase Pah1 to diacylglycerol (DAG), which is then used as a substrate by the diacylglycerol acyltransferase Dga1 or the phospholipid: diacylglycerol acyltransferase Lro1 to form TAG. Previous studies in *Yarrowia lipolytica* have shown that increasing levels of Gro-3-P by removing its utilization through the glycerol-3-phosphate dehydrogenase (Gut2) improved the TAG content 3-fold compared to the wt (Beopoulos et al., 2008). In *S. cerevisiae*, individual overexpression of either *PAH1* (Hung et al., 2016) or *DGA1* (Jensen et al., 2017; Runguphan and Keasling, 2014) resulted in a significant increase in TAG levels.

TAGs are stored in form of lipid droplets (LDs) together with sterol esters (SEs) (Kohlwein et al., 2013). When required by the cell, the acyl chains in these storage lipids can be cleaved by TAG lipases and SE hydrolases. Tgl3, Tgl4, and Tgl5 are members of the conserved patatin-domain-containing family of hydrolases (Athenstaedt and Daum, 2005) and simultaneous deletion of the respective genes has been proven to lead to an increase of TAG levels (Ploier et al., 2013). These studies indicate that TAG degradation mechanisms might counteract accumulation of high levels of these compounds, highlighting the importance of removing lipase activity and further fatty acid degradation such as fatty acid beta-oxidation.

The above-mentioned individual engineering strategies have been successful at increasing the TAG levels in *S. cerevisiae* and *Y. lipolytica*. However, there has not been any extensive study on exploring a combinatorial approach of all these strategies in a single strain. In this work, we developed a *S. cerevisiae* platform strain with high-level production and accumulation of TAGs. We therefore demonstrate the potential for *S. cerevisiae* to accumulate up to 25% of dry cell mass as TAGs without resorting to heterologous proteins.

2. Methods

2.1. Plasmid and strain construction

Strains, plasmids, links to plasmid maps, gRNA sequences and primers used in this study are listed in Table 1 and Additional File 1. Strain IMX581, and plasmids pROS10, pROS10, pMEL13 and pMEL10 were obtained from EUROSCARF (Frankfurt, Germany). Diagnostic primers and repair fragments were designed using the Yeastrestriction webtool (<http://yeastrestriction.tnw.tudelft.nl/>). Gene deletions were performed and verified as described in (Mans et al., 2015). DNA fragments for *DGA1* and *PAH1* were amplified from genomic DNA of CEN.PK 113–7D (provided by P. Kötter, University of Frankfurt, Germany). Sequences for *PGK1p* and *TEF1p* were amplified from pSP-G1 (Partow et al., 2010). Integrative plasmids were based on pCfB389 (XI-2-*loxP-KIURA3*) and

pCfB2055 (X-2-*loxP-KanMX4*), a derivative of pCfB353 (Jensen et al., 2014). The *KanMX4* cassette from pCfB2055 was subcloned with *XhoI*-*Sall* into pCfB389 to generate pMG95 (XI-2-*loxP-KanMX4*). The sequences for *PGK1p*, *TEF1p*, *PAH1* and *DGA1* were assembled into an integrative vector using pMG95 as backbone, generating pMG97. Sequences were assembled into integrative plasmids using CPEC (Quan and Tian, 2011). Construction of *HXT7p-ACC1*^{**} has been described elsewhere (Bergenholt et al., submitted). For integration, generated integrative vectors were linearized with *NotI* and transformed directly using a PEG/LiOAc technique (Gietz et al., 2007). Correct integration was confirmed using primers located up- and downstream of the integration sites. For construction of *HXT7p-ACC1*^{**} *PAH1* + *DGA1*, the *KanMX4* marker was consecutively removed by expressing the *cre* recombinase gene from pSH65 as described (Jensen et al., 2014).

2.2. Growth medium

S. cerevisiae strains with auxotrophies were grown on YPD plates containing 20 g·L⁻¹ glucose, 10 g·L⁻¹ yeast extract, 20 g·L⁻¹ peptone from casein and 20 g·L⁻¹ agar. *URA3* plasmid carrying strains were grown on selective growth medium containing 6.9 g·L⁻¹ yeast nitrogen base without amino acids (Formedium, Hunstanton, UK), 0.77 g·L⁻¹ complete supplement mixture without uracil (Formedium), 20 g·L⁻¹ glucose and 20 g·L⁻¹ agar. *KanMX4* plasmid carrying strains were grown on YPD plates supplemented with 200 mg·L⁻¹ Geneticin®. Shake flask cultivations were performed in minimal medium containing 20 g·L⁻¹ glucose, 5 g·L⁻¹ (NH₄)₂SO₄, 14.4 g·L⁻¹ KH₂PO₄, 0.5 g·L⁻¹ MgSO₄·7H₂O adjusted to pH 6. After sterilization, 2 mL·L⁻¹ trace element solution and 1 mL·L⁻¹ of vitamin solution were added. The composition of the trace element and vitamin solution has been reported earlier (Verduyn et al., 1992).

2.3. Shake flask cultivations

Biological triplicates were pre-cultivated in 5 mL minimal medium at 200 rpm and 30 °C for 18 h. Subsequently, the pre-culture was diluted into 15 mL minimal medium in a 100 mL shake flask to an OD₆₀₀ of 0.1. Shake flasks were incubated at 200 rpm and 30 °C for 72 h. A spectrophotometer (Genesis 20, Thermo Fisher Scientific, Waltham, MA, USA) was used to measure cell density at the end of the shake flask cultivations.

2.4. Lipid quantification

Samples for lipid analysis were taken at the end of the shake flask cultivations. The samples were centrifuged at 3000g for 5 min and the supernatant was discarded. Cell pellets were then washed with 50 mL deionized water, centrifuged at 3000g for 5 min and the supernatant was discarded. The pellets were kept at -20 °C for 10 min and then freeze-dried using a Christ alpha 2–4 LSC (Christ Gefriertrocknungsanlagen, Osterode, Germany). Lipids were extracted using microwave extraction and analyzed by HPLC-CAD using 10 mg of dried cell biomass as previously described (Khoormung et al., 2013).

Table 1
Strains used for this study.

Strain	Genotype	Parental strain	Reference
IMX581	<i>MATa ura3–52 can1::cas9-natNT2</i>	CEN.PK113–5D	Mans et al. (2015)
ADP	<i>MATa ura3–52 can1::cas9-natNT2 HXT7p-ACC1** PGK1p-PAH1 TEF1p-DGA1</i>	IMX581	This study
RF08	<i>MATa ura3–52 can1::cas9-natNT2 HXT7p-ACC1** PGK1p-PAH1 TEF1p-DGA1 tgl3/4/5Δ are1Δ</i>	ADP	This study
RF09	<i>MATa ura3–52 can1::cas9-natNT2 HXT7p-ACC1** PGK1p-PAH1 TEF1p-DGA1 tgl3/4/5Δ are1Δ pox1Δ</i>	RF08	This study
RF10	<i>MATa ura3–52 can1::cas9-natNT2 HXT7p-ACC1** PGK1p-PAH1 TEF1p-DGA1 tgl3/4/5Δ are1Δ pox1Δ gut2Δ</i>	RF09	This study
RF11	<i>MATa ura3–52 can1::cas9-natNT2 HXT7p-ACC1** PGK1p-PAH1 TEF1p-DGA1 tgl3/4/5Δ are1Δ pox1Δ gut2Δ pxa1Δ</i>	RF10	This study
RF12	<i>MATa ura3–52 can1::cas9-natNT2 HXT7p-ACC1** PGK1p-PAH1 TEF1p-DGA1 tgl3/4/5Δ are1Δ pox1Δ gut2Δ pxa1Δ faa2Δ</i>	RF11	This study

The neutral lipids stored in lipid droplets are normally composed of 50% TAGs and 50% sterol esters (SEs) (Kohlwein et al., 2013). An SE results from the esterification of an acyl-CoA with a hydroxyl group of a sterol, catalyzed by acyl-CoA: sterol acyltransferases encoded by the *S. cerevisiae* genes *ARE1* and *ARE2* (Yang et al., 1996). We proposed to remove sterol ester formation through deletion of *ARE1* and *ARE2* in order to obtain a larger fraction of the neutral lipids in form of triacylglycerols. Removal of the major lipase genes *TGL3*, *TGL4* and *TGL5* and deletion of *ARE1* resulted in the new strain RF08. Although *ARE2* has been reported to be the major SE synthase (Sandager et al., 2002), we faced major technical difficulties in removing the gene during several attempts and therefore this deletion was not included in the following strain construction. RF08 showed a 68% increase in TAGs levels compared to strain ADP, with a final concentration of 218 mg-gCDW⁻¹ (Fig. 2). The resulting strain did not show any change in the final OD in comparison to the reference strain (Fig. S2).

3.3. Removal of the fatty acyl-CoA peroxisomal transporter further increases TAG levels

In *S. cerevisiae*, acyl-CoA is degraded back to acetyl-CoA by the peroxisomal β -oxidation pathway, and inactivation of this pathway leads to improved production of acyl-CoA derived chemicals (Valle-Rodríguez et al., 2014). Removal of the first step of the β -oxidation encoded by *POX1*, resulted in the strain RF09 which accumulated 204 mg-gCDW⁻¹ of TAGs, a 9% decrease ($p > .05$) in comparison to RF08 (Fig. 2).

Next, we targeted the Gro-3-P utilization pathway by deleting the glycerol-3-phosphate dehydrogenase encoding gene *GUT2*. Gut2 catalyzes the conversion of Gro-3-P to dihydroxyacetone phosphate (DHAP) (Rønnow et al., 1993). Removal of *GUT2* resulted in the strain RF10, which accumulates TAGs at a level of 222 mg-gCDW⁻¹, similar to the level of RF08 (Fig. 2).

We deleted *PXA1* encoding a subunit of the peroxisomal transporter involved in the import of acyl-CoA (Shani et al., 1995). The resulting strain RF11 reached a TAG level of 254 mg-gCDW⁻¹, a 96% increase in comparison to the ADP strain (p value $< .05$), and a 14% increase in comparison to RF10 (p value $< .05$) (Fig. 2). The TAG titers produced by RF11 of 1.76 g-L⁻¹ from 20 g-L⁻¹ glucose translates to 27.4% of the maximum theoretical yield when considering the theoretical yield to be 0.32 gTAG-gGlucose⁻¹ (Table 2) (Caspeta and Nielsen, 2013). Notably, the titer achieved in RF11 is the highest reported in *S. cerevisiae* (Table S1). Finally, we deleted a gene encoding a peroxisomal fatty acid activating enzyme, *FAA2*, leading to strain RF12 strain. The final strain accumulated a similar level of TAG (248 mg-gCDW⁻¹) compared to RF11.

4. Discussion

Lipid metabolism in eukaryotes is complex due to its crosstalk with essential cellular processes, such as synthesis of signaling molecules and cellular membranes. For this reason, it is tightly regulated at several levels, often making it a difficult target for metabolic engineering.

Here, we started by implementing a typical push-and-pull strategy towards TAG accumulation. For that, engineering of *Acc1* at the transcriptional and phosphorylation level was a fundamental step due to the importance of this enzyme in controlling fatty acid metabolism. The initial approach of overexpressing genes encoding an *Acc1* with two removed phosphorylation sites, together with *Pah1* and *Dga1* resulted in TAG accumulation levels up to 12% of dry cell biomass, more than 10-fold above the levels in the reference strain. A similar strategy of overexpressing *ACC1* and *DGA1* in *Y. lipolytica* reached to a lipid content of 41.4% (Tai and Stephanopoulos, 2013). Previous studies based on individual expression of these three genes have reported similar titer improvements, with more pronounced effect for *Acc1* (Hofbauer et al., 2014; Hung et al., 2016; Runguphan and Keasling, 2014). Acetyl- and malonyl-CoA constitute the building blocks of fatty-acid biosynthesis, and these results show the importance of the *Acc1* level and activity being a central metabolic knot of the lipid metabolism in *S. cerevisiae*.

Next we removed most lipase activity and part of the sterol ester synthesis activity and observed an TAG increase of up to 80%, reaching close to 22% of the dry cell weight. These results strongly indicate that in the ADP strain, lipid homeostasis mechanisms are counterbalancing TAG accumulation with TAG hydrolysis, therefore limiting further production. Supporting this, previous reports have shown that lipase activity at transcriptional and protein level is affected by neutral lipid levels (Klein et al., 2016). Based on similar experiments realized in *S. cerevisiae*, we speculate that the removal of the lipases has had the major role in increasing TAG levels (Sandager et al., 2002; Athenstaedt and Daum, 2003).

In contrast with previous results obtained in *Y. lipolytica*, the disruption of the glycerol consumption gene, *GUT2*, did not lead to a significant increase in TAGs (Dulermo and Nicaud, 2011). Here, we hypothesize that the flux towards TAG biosynthesis is not constrained by the availability of Gro-3-P. This diverges from studies made in *Y. lipolytica*, where TAGs can accumulate to 80% of the cells' dry weight. This high lipid content in *Y. lipolytica* might indicate that fatty acid synthesis exceeds Gro-3-P availability in this organism, making Gro-3-P a limiting factor in TAG synthesis, which does not seem to be the case in *S. cerevisiae*. While not tackled in this study, recent studies have reported an improvement of TAG levels when overexpressing heterologous GPAT and LPAT genes, which suggests a potential additional engineering strategy reach higher TAG titers (Wei et al., 2017). Overexpression of the endogenous GPAT and LPAT genes could be considered as well (Fig. 1).

Additionally, neither the disruption of the first reaction of the β -oxidation, catalyzed by *Pox1*, nor removal of the import activation of FFAs into the peroxisomes catalyzed by *Faa2*, affected the TAG levels. In *S. cerevisiae*, in normal condition, a wild-type strain only produces very low levels of FFAs, as they are directly and quickly re-activated by fatty acyl-CoA synthetases to fatty acyl-CoAs (Zhou et al., 2016). Although previous studies have shown that *POX1* disruption increased TAGs in *S. cerevisiae* and *Y. lipolytica* (Valle-Rodríguez et al., 2014; Dulermo and Nicaud, 2011), the removal of the major TAG lipases further decreased the supply of FFAs, which could explain the null effect of *POX1* disruption. Additionally, the null effect from removing

Table 2
Yield calculations.

Strain	mgTAG/gDCW	Biomass (gDCW/L)	Titer (gTAG/L)	Yield (gTAG/gGLuc)	%Theoretical Yield*
IMX581	7.45 \pm 1.51	7.30 \pm 0.07	0.05 \pm 0.01	0.003 \pm 0.001	0.8% \pm 0.2
ADP	129.30 \pm 10.05	7.90 \pm 0.26	1.02 \pm 0.10	0.051 \pm 0.005	16.0% \pm 1.6
RF08	217.78 \pm 12.43	7.95 \pm 0.18	1.73 \pm 0.10	0.087 \pm 0.005	27.0% \pm 1.6
RF09	201.23 \pm 10.18	8.00 \pm 0.22	1.61 \pm 0.05	0.080 \pm 0.003	25.1% \pm 0.8
RF10	221.86 \pm 20.77	6.74 \pm 0.17	1.50 \pm 0.17	0.075 \pm 0.009	23.4% \pm 2.7
RF11	254.02 \pm 19.87	6.91 \pm 0.04	1.76 \pm 0.15	0.088 \pm 0.007	27.4% \pm 2.3
RF12	247.87 \pm 10.13	6.73 \pm 0.27	1.67 \pm 0.12	0.083 \pm 0.006	26.1% \pm 1.8

* Assuming 0.32 g/g as theoretical maximum yield.

FAA2 further confirms this hypothesis. Presumably, FAA2 disruption would have allowed more cytosolic FFA substrates to be converted to fatty acyl-CoA, ultimately resulting in more TAGs.

Finally, disruption of the peroxisomal fatty acyl-CoA transporter gene, *PXA1*, resulted in a 14% ($p < .05$) increase of the TAG level. *Pxa1* transports fatty acyl-CoA to the peroxisome which ultimately decreases the levels of available substrate for TAG biosynthesis. Altogether, these results highlight a potential limitation in cytosolic acyl-CoA availability for TAG biosynthesis.

We envision that other aspects of lipid metabolism can be engineered for a higher level of TAG accumulation. These include improvements in fatty acid biosynthesis, such as improving FAS activity, further enhancing the supply of acetyl-CoA and malonyl-CoA, or NADPH cofactor balancing (Chen et al., 2013; Yu et al., 2017; Qiao et al., 2017; Hung et al., 2016).

In conclusion, the strains generated in this study provide good platforms on which further development can be implemented to generate efficient TAG-producing yeast cell factories. Furthermore, through generation of these strains we gained new insight into the regulation of TAG biosynthesis in the important model organism *S. cerevisiae*.

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Author's contribution

R.F, V.S, F.D and J.N conceived and designed the project. R.F, P.G.T and M.G performed the experiments. R.F. and P.G.T wrote the manuscript. All authors read and approved the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.meten.2018.01.002>.

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